Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

Kurt W. Kohn, MD, PhD
Scientist Emeritus
Laboratory of Molecular Pharmacology
Developmental Therapeutics Branch
National Cancer Institute
Bethesda, Maryland
kohnk@nih.gov

CHAPTER 2: Anti-cancer drugs that attack DNA at a critical site on guanine

The Guanine-O6 Story: a DNA Target and a Brain Cancer Treatment

Although the nitrogen at guanine position 7 is the most readily alkylated site on DNA, some alkylating drugs are potent enough to attack also the oxygen at guanine position 6, where the impact on the cell is much greater, leading to mutations and eventual cell death. As a reminder, "alkylation" means that a chemical group, such as methyl, ethyl, or chloroethyl, becomes bound tightly (covalently) to the atom that is "alkylated", such as the nitrogen atom at position 7 or the oxygen atom at position 6 of guanine (Figure 2.1).

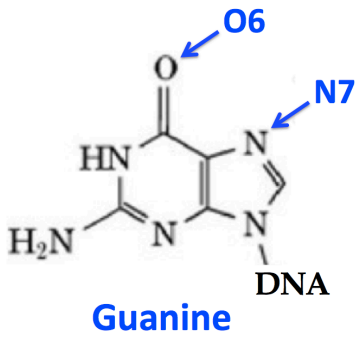


Figure 2.1. The major alkylation sites on DNA attacked by the drugs discussed in this chapter are on the nitrogen atom at position 7 (GN7) and the oxygen atom at position 6 of guanine (GO6). The focus is on attack at GO6.

The MGMT story and its impact on cancer treatment.

The alkylating agents discussed in the preceding chapter, as well as the platinum drugs to be discussed in the next chapter, all attack DNA primarily at the nitrogen at guanine N7 (GN7). However, there is another class of alkylating agents, which additionally attack DNA at the oxygen at guanine position O6 (GO6). What is special about alkylation at GO6 is that it drastically affects the pairing of guanine with cytosine in the DNA double helix (Figure 2.2): alkylation at GO6 allows the guanine to pair just as well with thymine as with cytosine, which is apt to cause a mutation. GO6 alkylations cause a host of troubles for the cell, as we shall see.

Since some carcinogens can alkylate DNA at guanine-O6, a special enzyme, called MGMT (for methylguanine-methyltransferase), has evolved to quickly and efficiently remove such alkylations before they can cause trouble. Since MGMT merely removes the offending alkyation, the normal guanine is regenerated. Hence, this repair is error-free, in contrast with most other DNA repair processes, which are prone to making mistakes (Figure 2.3).

Inadequate MGMT may cause a mutation that is an early step in the development of cancer. In colon cancer, for example, MGMT is suppressed (by promoter methylation; see below) in about 40% of cases and may be an early event in the development of these cancers (Fornaro et al., 2016).

Another effect of low MGMT in cancer, however, is to make those cancers vulnerable to drugs that alkylate DNA at GO6. The lack of adequate amounts of MGMT to remove the alkylations damages or kills the cancer cells. Some cancers do have low MGMT levels and therefore tend to be sensitive to GO6-alkylating drugs. Herein lies an opportunity for therapy targeted to tumors that have low levels of that DNA repair enzyme (Hegi and Stupp, 2015).

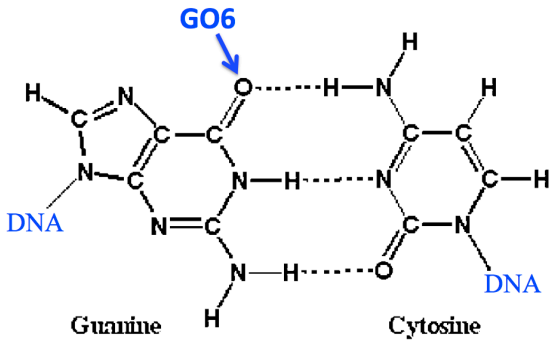


Figure 2.2. A guanine:-cytosine base-pair in DNA. If the O6 position of guanine is alkylated (e.g., methylated), the hydrogen-bonds that holds the G:C base pair together are disrupted. (A hydrogen bond is a weak bond between H and O, or between H and N, indicated by dashed lines.) The O6-alkylated guanine then can base-pair with thymine rather than cytosine. The result, after DNA replication, is

that the G:C base pair is replaced by an A:T base pair, which may change an amino acid in a protein.

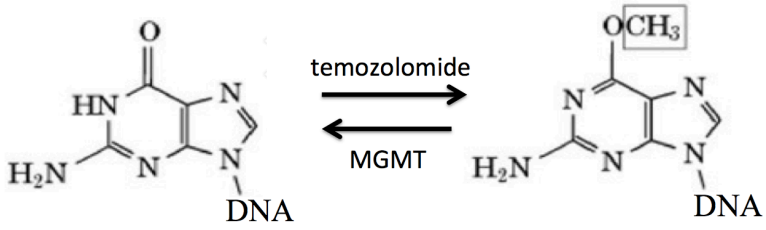


Figure 2.3. Temozolomide alkylates guanine by adding a methyl group (CH_3) to the GO6 position. The DNA repair enzyme, MGMT, removes methyl groups, as well as other alkylations, from that position and regenerate normal guanine.

The MGMT story began in 1980, with a groundbreaking observation by Rufus Day, a former colleague at the National Cancer Institute (Day et al., 1980a; Day et al., 1980b). His investigation was inspired by the work of Paul Kornblith, a neurosurgeon colleague of ours at NCI, who had found that cells derived from brain tumors from different patients varied greatly in their sensitivity to BCNU/carmustine (a GO6-trageted DNA crosslinking drug that will be discussed later in this chapter) (Kornblith and Szypko, 1978). Following up on that finding, Rufus demonstrated that cells from some tumors were abnormally sensitive to GO6-targeted alkylating agents, because they had a defect in a DNA repair process.

In that work, Rufus used an assay based on the fact that DNA repair-deficient cells cannot support the growth of a DNA-damaged adenovirus. He first treated the adenovirus with a GO6-targeted alkylating agent, so that the virus could grow only in cells that could repair the GO6 alkylations. Using that assay, he showed that cells whose DNA repair system was defective, were unusually sensitive to being killed by GO6-targeted alkylating agents. In other words, the cells that could not repair the guanine-O6-methylated virus, could not repair their own DNA either; therefore they died upon treatment with relatively low concentrations of those drugs (Day and Ziolkowski, 1979; Day et al., 1980a; Day et al., 1980b).

Rufus surmised correctly that there was a phenotype, which he called *Mer*$^-$ for "methylation repair minus," that made some tumors abnormally sensitive to alkylating agents of the GO6-alkylating type (Day et al., 1980a). The reason he called that phenotype *methylation* repair deficient was because the agents he used added a methyl groups to O6-guanine on DNA, and the presumed repair involved removal of those methyl groups from DNA guanine. The high drug sensitivity was present only for alkylating agents that targeted GO6 and only to cells that were unable to remove the GO6 alkylations efficiently. In 1983, Dan Yarosh, working with Rufus Day, confirmed that *Mer*$^-$ human tumor cells are unable to repair O6-methylguanine in DNA by demethylation (Yarosh et al., 1983).

In a companion paper that followed Rufus Day's in *Nature*, Leonard Erickson in my laboratory demonstrated that, after treatment with the GO6-targeted DNA crosslinking drug, chloroethylnitrosourea, the repair-deficient (Mer^-) cells, were not only consistently killed by low concentrations of the drug, but also sustained higher levels of DNA inter-stand crosslinks (Erickson et al., 1980). That result was confirmed by Eric Sariban, working with Len Erickson, for human cell strains derived from glioblastoma tumors (Sariban et al., 1987). (How chloroethylating drugs produce DNA crosslinks, while methylating agents do not, will be explained later in this chapter.)

The enzyme that specifically removes alkylations from DNA guanine-O6 sites, as well as the gene that codes for it, were soon identified. The gene is called "MGMT" for "O6-methylguanine-methyltransferase," but it (that is, its protein product) removes a variety of GO6 alkylations, not only methyl groups. It turned out that the MGMT gene is turned off ("silenced") in the sensitive (Mer^-) cells; they are sensitive, because they cannot remove the GO6 alkylations.

The cause of the MGMT silencing was also soon discovered. The gene is silenced, because it is "methylated"; this methylation is not on guanine; rather, it is a normal gene-regulation process in which cytosines in the vicinity of gene start regions ("promoter region") in DNA are methylated. Thus, when the MGMT gene is promoter-methylated, the enzyme that removes alkylations from the guanine-O6 position is not produced (or very little is produced).

Two classes of anti-cancer drugs were found to alkylate guanine-O6 on DNA: (1) chloroethylnitrosoureas, which add chloroethyl groups at GO6 and form crosslinks, and (2) temozolomide and dacarbazine, which add methyl groups at GO6 and do not form crosslinks.

The temozolomide story and the treatment of brain cancer.

Temozolomide was the most notable advance in the treatment of the highly malignant brain tumor, glioblastoma up to the time of this writing (Ajaz et al., 2014; Stupp et al., 2005). This "blockbuster drug" came at the pinnacle of a series of compounds investigated at Aston University in Birmingham, UK, beginning in an antitumor pharmacology group organized by John Hickman and Andy Gesher (Stevens and Newlands, 1993).

Figure 2.4. Malcolm Stevens, developer of temozolomide (Sansome, 2009). (Photograph from Chemistry World, 2009.)

The temozolomide story began in 1978, when Robert Stone, a PhD student, joined Malcolm Stevens' drug discovery laboratory at Aston University. Stevens' instruction to Stone was brief and open-ended: "make some interesting molecules" (Sansome, 2009) (Figure 2.4); the *modus operandi* of the laboratory was to synthesize creative and potentially useful organic compounds. Stevens evidently felt that allowing a talented young mind freedom of action could lead to something out of the box, as indeed it did.

Stone was interested in ring compounds with several nitrogens, including a nitrogen atom at the junction of 2 rings (a so-called bridgehead nitrogen), and he had read about a new route to the synthesis of some compounds of that sort. With that start and Stevens' chemical insights, they came up with a brand new 2-ring system (called imidazotetrazinone) that had never been seen before (Sansome, 2009).

They knew they were heading into the realm of alkylating agents, with some resemblance to dacarbazine (Figure 2.4), which has 3 nitrogens in a row, although not in a ring, and which was in use for the treatment of melanoma. Of concern was that, because so many alkylating agents had already been tried and their problems were well known, such drugs had lost much of their luster. In fact, when they finally came up with temozolomide, despite its remarkable effectiveness against almost all mouse tumors tested, Stevens had a hard time convincing clinical researchers to put it in clinical trial. An advantage that may have helped its acceptance for clinical trial was that, as a pro-drug, it could conveniently and safely be taken by mouth. Since it was lipid soluble and had a chemical structure that could generate a nitrosourea-like moiety, it was reasonable to test it against glioblastoma.

Stevens and Stone were not happy with the name, temozolomide that the manufacturer assigned to it, because it gave no hint of its chemical nature or origin; they wanted to call it "azolastone," which would combine "azo" for nitrogen, "Aston"

for the name of the University where it was made, and "Stone" for the name of the student who made it. That creative name, however, did not prevail, because the manufacturer feared it could be confused with the name of an antihistamine then in use, and perhaps because an unkind person might come up with "azo-last-one" (Sansome, 2009).

Along the way to temozolomide, a drug (mitozolomide) having a chloroethyl in place of the methyl, and therefore a DNA crosslinker, had been in clinical trial, but was dropped because of excessive toxicity (Stevens and Newlands, 1993). Replacing the choloethyl with a methyl in temozolomide did not seem a promising move, but was motivated by its effectiveness in mice. Despite the fragile rationale, clinical trial, after an adjustment of dosage schedule, revealed temozolomide's surprising potential as an antitumor drug.

How does temozolomide work?

Temozolomide was found to be a “pro-drug” that is inactive until converted in the liver to form the active drug (Figure 2.5). Moreover, it was one of the few anti-cancer drugs able to penetrate the “blood-brain barrier” to get into the brain and have access to tumors in the brain. Temozolomide proved so effective that it, combined with radiation, became the standard treatment for glioblastoma (after surgery, where possible) (Stupp et al., 2015).

Temozolomide (after activation) was found to methylate guanine-O6 positions in DNA and did not form crosslinks. However, although GO6 methylation can kill cells, it was also noted for *producing* mutations and cancer. But those nasties took many years to show up, whereas glioblastoma patients, even with the best available therapy, rarely survived that long.

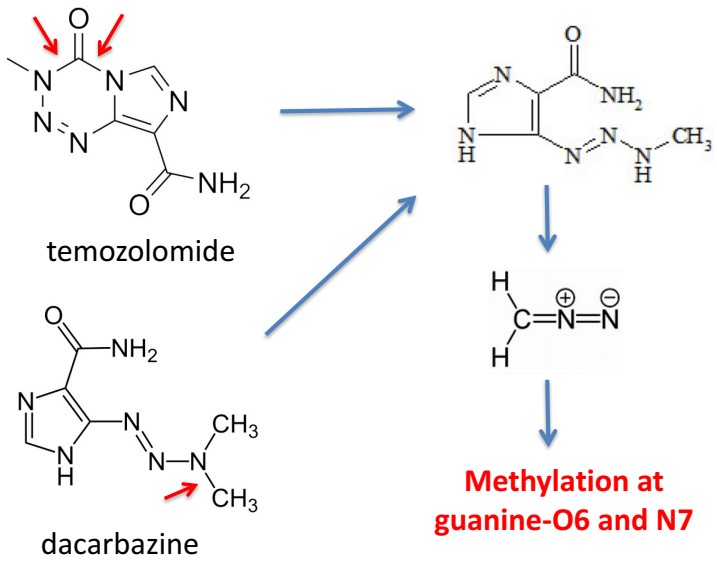


Figure 2.5. Temozolomide (upper left) is activated by enzymes in the liver that cleaves of the bonds indicated by red arrows. Dacarbazine (lower left) is activated by a liver enzyme that cleaves of a bond (red arrow) to remove a CH_3 group. The activations of both drugs yield the structure on the upper right, which decomposes spontaneously to form a highly reactive molecule potent enough to methylate DNA guanines at O6 as well as at N7.

After Rufus Day, Leonard Erickson and their colleagues had reported that deficiency in MGMT enhanced the response of cancer cells to GO6-targeted drugs, such as temozolomide, dacarbazine, and chloroethylnitrosoureas (BCNU/carmustine and CCNU/lomustine), their findings were confirmed in clinical studies, which were made possible by development of suitable assays (Belanich et al., 1996; Esteller et al., 2001; Esteller et al., 2000b; Hegi et al., 2005) (Figure 2.6), as well as later using a more precise assay method (Barault et al., 2015) (Figure 2.7).

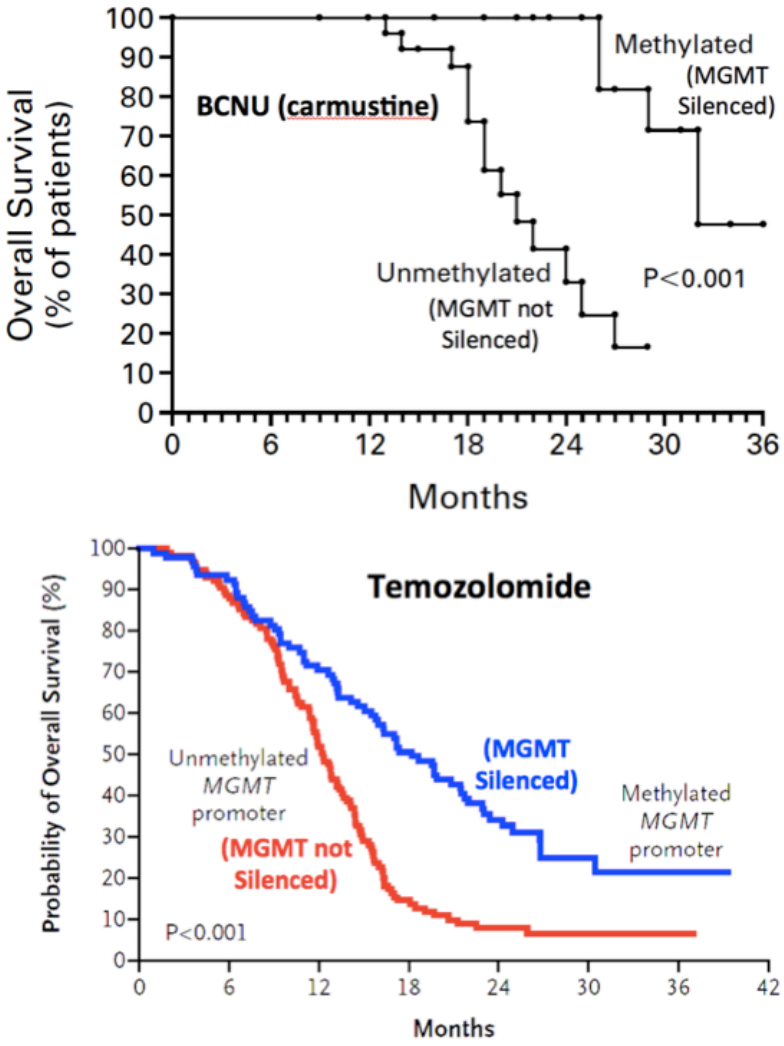


Figure 2.6. Patients treated for malignant brain tumors (glioblastomas) survived longer if their tumor's MGMT gene was not functioning (silenced). The patients were treated with BCNU (carmustine, top (Esteller et al., 2000a)) or temozolomide (bottom, (Hegi et al., 2005)). (The BCNU and temozolomide studies cannot be compared with each other, because they were carried out at different times in different universities using different protocols.) From the New England Journal of Medicine.

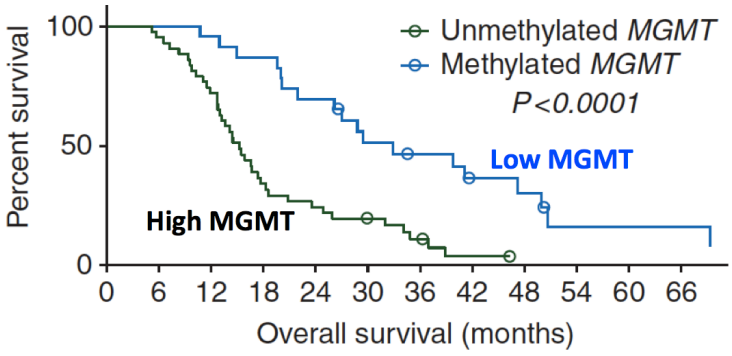


Figure 2.7. Increased survival of temozolomide-treated glioblastoma patients whose tumors had low levels of MGMT, compared with those whose tumors had high levels. Low level of MGMT production was indicated by promoter-methylation of the MGMT gene (blue curve). This study, carried out with a more precise method, confirmed the results in Figure 2.6 (Barault et al., 2015).

Temozolomide treatment of brain cancer patients.

In 2000, Esteller and coworkers showed that the GO6-targeted DNA crosslinking drug, BCNU (carmustine), produced more benefit to glioblastoma patients whose tumors' MGMT genes were inactive due to DNA methylation (Esteller et al., 2000a) (Figure 2.6). In 2005, Monika Hegi and Roger Stupp reported similar results for temozolomide; they estimated that the MGMT gene was silenced (by promoter methylation) in the tumors of 45% of their malignant glioblastoma patients, and that it was only those patients who benefited from treatment with temozolomide: they lived longer, whereas patients with tumors whose MGMT genes were not silenced had little or no benefit from the drug (Hegi et al., 2005). These studies eventually defined the standard of care for newly diagnosed glioblastomas.

In 2015, it was again reported that temozolomide was effective only against glioblastomas whose MGMT genes were silenced (Barault et al., 2015; Lombardi et al., 2015) (Figure 2.7). Similar conclusions were reported also for patients with colon cancer (Fornaro et al., 2016; Pietrantonio et al., 2015).

However, despite the accumulating evidence for the importance of MGMT status, it was some time before MGMT status was regularly considered in determining whether a patient's brain tumor was likely to respond to temozolomide. Glioblastoma patients continued to be treated with temozolomide, regardless of their tumor's MGMT status. In 2015, Hegi and Stupp published an article in the New England Journal of Medicine, asking why that was the case (Hegi and Stupp, 2015). Why were more than half of glioblastoma patients continuing to be treated with a drug that the MGMT test indicated would not benefit them? The authors pointed out that, by omitting temozolomide in the treatment of patients with MGMT-active

tumors, there would have been an opening to test innovative therapies for those patients who were unlikely to be benefited by temozolomide.

Thus, research emphasis on glioblastoma brain cancer continued to aim in the direction of the conventional idea that the main barrier to successful chemotherapy was drug-resistance of the tumor, and that the obvious thing to do was to overcome the cause of the resistance. Drugs were therefore developed to inhibit the MGMT enzyme. The clinical results of combining temozolomide with an MGMT inhibitor were disappointing, which was not at all surprising, because the inhibition of MGMT also sensitized critical normal tissues to the drug. This misguided clinical research direction delayed the opportunity to select the treatment that would be most likely to increase survival and minimize toxicity in glioblastoma patients. (Since I was engaged in the pre-clinical research, a disclosure is needed. I had argued strongly for emphasis on MGMT status and against the use of MGMT inhibitors. But to no avail, perhaps because, despite my efforts, I lacked the ability to make the argument convincing enough.)

Recent findings indicate that MGMT status is important for treatment decisions for the less malignant gliomas brain tumors as it is for the highly malignant glioblastomas (Figure 2.8) (Bell et al., 2018).

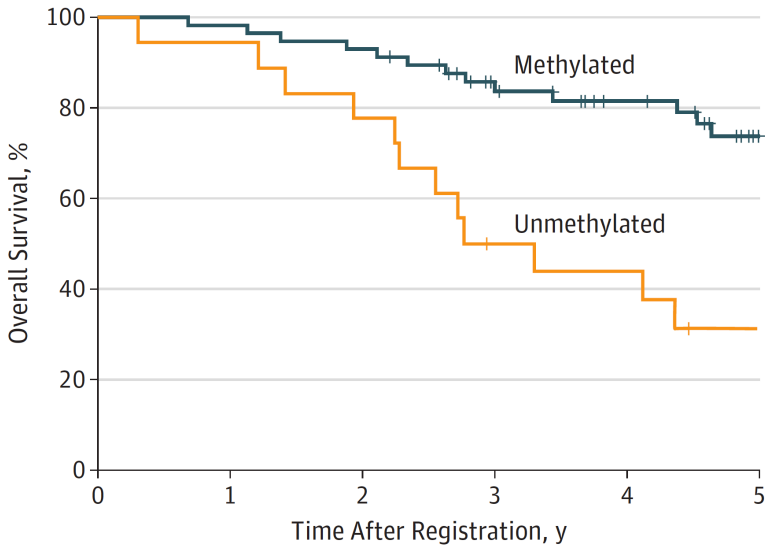


Figure 2.8. A recent study showing that low-grade gliomas whose MGMT gene is silenced by DNA methylation respond better to temozolomide plus radiation than do gliomas whose MGMT gene is unmethylated and therefore not silenced. Thus, MGMT status is important for these less malignant brain cancers, as well as for the highly malignant glioblastomas (Bell et al., 2018).

DNA mismatch repair and a surprise

If DNA is replicated through an GO6-alkylated guanine before MGMT has repaired it, a more sinister DNA repair process comes into play. Called "mismatch repair," it detects and tries to repair places in DNA that are not properly base-paired. In the case of GO6-methylated DNA, however, it back-fires and kills more cells than it helps. It has in fact turned out to be an important factor in clinical response to temozolomide. Surprisingly, patients whose tumors have low mismatch repair capacity (indicated by low content of the mismatch repair enzyme, MSH2, survive significantly longer than those whose tumors are high in MSH2 (Figure 2.9). The repair process, instead of making the tumors resistant to temozolomide, enhances the killing of the tumor cells. This effect is particularly prominent in cancers that are low in MGMT, because those cancers are likely to have persistent O6-alkylated guanines (because the MGMT that would have reversed them is lacking).

The O6-alkylated guanines look like a base-pair mismatch to the mismatch repair system, which however is often unable to repair them and instead produces more DNA damage

Here is how it is that happens. When DNA is replicated through an O6-methylated guanine, the replication machinery often inserts a thymine instead of a cytosine in the newly synthesized strand to pair with the O6-methyl-guanine. The resulting methyl-O6-guanine : thymine base-pair is detected as a DNA defect by the mismatch repair system, which proceeds to remove a section of one of the strands that includes the now mis-paired methyl-guanine or thymine. A DNA repair synthesis system then comes into play to replace the DNA section that the mismatch repair system has removed; but the repair synthesis again often pairs a thymine with the O6-methyl- guanine. This futile repair cycle continues until it comes to the attention of another surveillance system that concludes that it's time to give up trying to repair this mess, and signals the cell to commit suicide by apoptosis (McFaline-Figueroa et al., 2015). When that happens in a tumor cell, it's good news. The surprise therefore is that the mismatch repair system assists in killing the MGMT-deficient cancer cells.

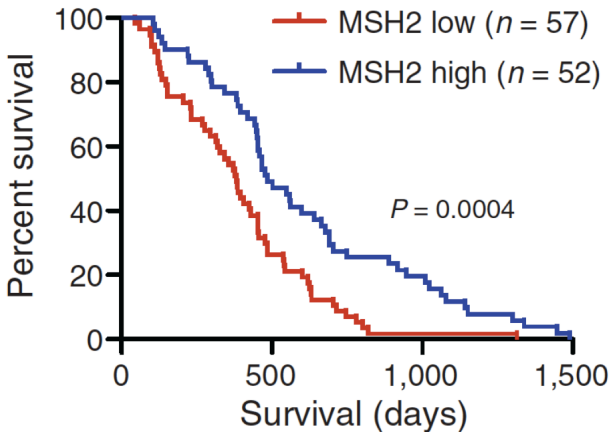


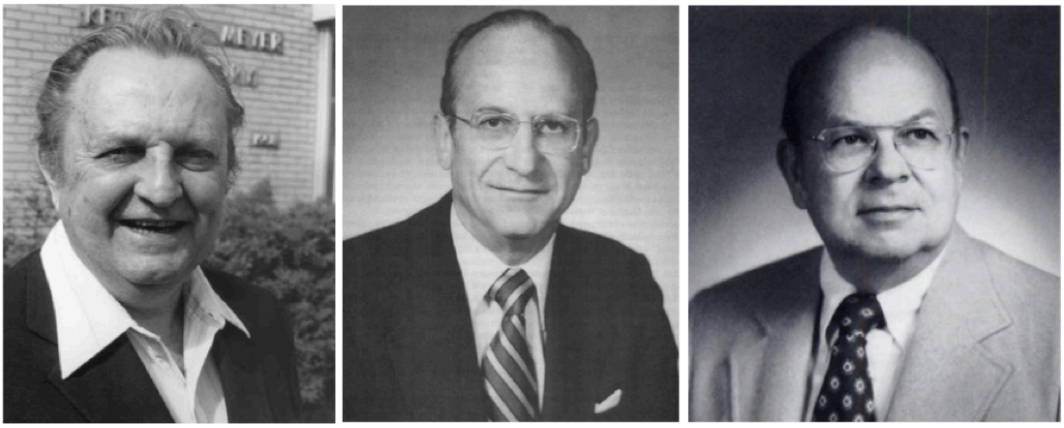
Figure 2.9. Temozolomide-treated glioblastoma patients whose tumors have high DNA mismatch repair capacity (blue curve) surprisingly survive longer than those with tumors low in this repair capability (red curve). Mismatch repair capacity was gauged by the level of the MSH2 protein in the tumor (McFaline-Figueroa et al., 2015).

The story of the chloroethylnitrosoureas: promise and disappointment.

One of the most promising leads to come out of the early years of the NCI's anti-cancer drug screening program were the chloroethylnitrosoureas; these drugs aroused strong interest because they were found to be highly effective against tumors in mice and, particularly, because of their unusual effectiveness against tumors in the brain. True to the complexity of their name, however, they were fraught with several concurrent chemical reaction paths, which frustrated efforts to attain a consistent balance between therapeutic and toxic effects. Toxicity tended to be delayed, unpredictable and difficult to manage. Although the chloroethylnitrosoureas are more potent and able to crosslink DNA, they were not any more effective than temozolomide in the treatment of malignant brain tumors (glioblastomas). The story of the rise and decline of the chloroethylnitrosoureas is a good example of how chemistry and therapy interact, but the story may not yet be over.

In 1972, Joseph Burchenal and Steven Carter, in their review of available anti-cancer drugs, listed two chloroethylnitrosoureas, BCNU and CCNU (also known as carmustine and lomustine, respectively), as "agents of proven clinical value" (Burchenal and Carter, 1972). Unfortunately, their clinical benefit turned out to be less than many had hoped.

The story began in 1960 at the Southern Research Institute in Birmingham, Alabama, with the work of three remarkable cancer researchers, who became noted for many contributions to experimental cancer chemotherapy: Howard E. Skipper, Frank M. Schabel, and John A. Montgomery (Figure 2.8).



Howard E. Skipper
1915-2006

Frank M. Schabel, Jr.
1918-1983

John A. Montgomery
1924-

Figure 2.10. Leaders in experimental chemotherapy research that led to the development of the chloroethylnitrosoureas BCNU (carmustine) and CCNU (lomustine).

Howard Skipper (1915-2006) was one of the many researchers and clinicians who were engaged in the mustard gas and nitrogen mustard studies during World War II (see Chapter 1), and who were eager to apply their new knowledge to cancer, becoming leaders in anti-cancer drug development and cancer chemotherapy. A biochemist by training, he served in the U.S. Army Chemical Warfare Service, which was led by Cornelius P. Rhoads, who also organized the first anti-cancer trials of nitrogen mustard. Rhoads later recommended him to start a biochemistry department at the new Southern Research Institute in Birmingham, Alabama, where Skipper established a world-famous experimental cancer research program (Simpson-Herren and Wheeler, 2006). He became well-known for his precise models of cancer cell growth in mice, which were fundamental concepts later used by clinician reseachers to design drug dosage scheduling and combinations, including those that led to the cure of childhood acute leukemia and Hodgkin's lymphoma (DeVita Jr., 2015).

Frank M. Schabel (1918-1983) worked closely with Skipper at Southern Research Institute to develop important principles of cancer chemotherapy; their names were associated together in some of their most notable contributions. John Montgomery in his 1982 Cain Memorial Award Lecture of the American Association for Cancer Research described Dr. Schabel as "the most able cancer chemotherapist in the world" (Montgomery, 1982).

Dr. Schabel's untimely death while at the helm of cancer chemotherapy research was an unfortunate setback. On the morning of August 30, 1983, Dr. Schabel had taken his place in the front row of a conference room in the Hofburg Palace in Vienna, Austria, at the 13th International Congress on Chemotherapy. He was scheduled to give the second talk that morning. A few minutes before the start of the

session, he had a sudden cardiac arrest from which the physicians in the room were unable to revive him (Freireich, 1984). His colleagues and friends were devastated and cancelled the session. I was at a different session at the time and was looking forward to discussing the nitrosourea problem with him, when later that morning I was shocked to hear from a stunned J Freireich what had happened. Frank Schabel's crystal clear analyses, and his -- I would say in the spirit of Vienna, "gemuetlich" -- style of conversation that exuded collegial friendship, were always enjoyable and enlightening, and I deeply regretted his untimely passing.

John A. Montgomery (b. 1924) joined the Southern Research Institute in 1952, and served as Director of Organic Chemistry Research from 1956 to 1986. He spearheaded the development of several new classes of anti-cancer drugs, including the chloroethylnitrosoureas, and was highly regarded for his opinions and judgment that contributed much to the drug development program of the National Cancer Institute.

The chloroethylnitrosourea story dates from 1961, when Howard Skipper and Frank Schabel reported a systematic study of drug effects in mouse leukemia L1210, in which they noted that methylnitrosourea, one of the many compounds they studied, extended the life-span of the leukemia-bearing mice even when the leukemia cells were growing in the brain (Skipper et al., 1961). That was unusual, because few, if any, of the previous promising drugs were able to cross the blood-brain barrier. Therefore, they modified the methylnitorosourea molecule to try to increase its potency while hopefully retaining its activity against tumors in the brain. The most promising of these were compounds that had a chloroethyl group ($ClCH_2CH_2$-) attached to the nitrosourea moiety (Figure 2.11).

The first of that series to be further investigated was BCNU (carmustine). BCNU was made with two chloroethyl groups, because it was originally designed to resemble nitrogen mustard. However, only the chloroethyl attached to the nitroso (-N=O) end of the molecule is important; the other turned out to be irrelevant and was replaced without loss of activity by a non-reactive cyclohexyl group in the next of the series to be investigated, CCNU/lomustine (Figure 2.11).

Two years later, in 1963, Schabel and Skipper reported that BCNU had marked activity against L1210 leukemia in mice and that it appeared to be a new type of alkylating agent with an anti-tumor profile different from the nitrogen mustards (Schabel et al., 1963). Particularly encouraging was that the drug, as hoped, was effective even when the leukemia cells were inoculated into the brain. That was remarkable because other drugs did not get into the brain and were ineffective against brain tumors. The researchers realized that BCNU was lipid soluble (that is, it dissolves in fat), and therefore could penetrate the fatty substance of the blood-brain barrier.

Interest in chloroethyl-nitrosoureas mounted further when, in 1977, John Montgomery reported that those drugs were highly active against advanced Lewis

Lung cancer in mice, a tumor that was notoriously resistant to treatment with other drugs; and, most remarkably, some of the mice with advanced Lewis Lung tumors were even cured (Montgomery et al., 1977).

Because of their remarkable effectiveness against malignant tumors in mice and their ability to cross the blood-brain barrier, chloroethylnitrosoureas, particularly BCNU/carmustine and CCNU/lomustine, were used to treat patients with malignant brain tumors, such as glioblastomas. Their effectiveness, however, was limited by their toxic side effects, which were delayed, unpredictable, and difficult to manage. Therefore, the chloroethylnitrosoureas were largely replaced by the less potent, but more effective, temozolomide, which could be given orally, and whose toxicity was easier to manage. The standard treatment for glioblastoma then became surgery, radiation, and temozolomide. Despite intensive therapy, however, patients generally survived for little more than one year. BCNU, given to patients after relapse, had little benefit (Reithmeier et al., 2010).

As an alternative to temozolomide in the treatment of glioblastoma, a 3-drug combination was tried, consisting of CCNU/lomustine plus procarbazine (an early variant of dacarbazine) and vincristine (discussed in Chapter 17), but without notable benefit. CCNU by itself increased survival by no more than a few months and then only in a minority of patients. Adding other drugs, such as procarbazine or vincristine, to the regiment yielded no further benefit. The outlook was bleak indeed (Ajaz et al., 2014).

Why they failed: too many reaction paths?

With such remarkable anti-tumor effect in mice, why did the chloroethylnitrosoureas fail in cancer patients? We still don't know. But it might have had something to do with the multiple and complicated reactions of which these drugs were capable. Because the drugs were so effective against mouse tumors, enormous effort went into unraveling their chemistry and their mechanism of action, in hope of finding out how to separate their toxicity away from their anti-cancer activity (Habraken et al., 1990; Kohn, 1977, 1981; Li et al., 2003; Ludlum, 1997; Matijasevic et al., 1993; Sariban et al., 1984). The studies pointed to DNA crosslinks, mainly of the inter-strand type, as the major cause of the cell killing. Research therefore focused on bringing to light the chemical reaction paths that led to the crosslinking.

Chloroethylnitrosoureas, their complicated reactions, and DNA crosslinking.

DNA inter-strand crosslinks are the most likely cause of cell killing by chloroethylnitrosoureas, but there are several chemical pathways that could damage cells in a variety of ways. The chloroethylnitrosouea molecule is inherently unstable and breaks apart spontaneously into 2 chemically reactive pieces (Figure

2.11). The left half of the molecule forms a powerful alkylating agent (chloroethydiazohydrazide) that can go on to form inter-strand crosslinks, either by attack at guanine-N7 (like nitrogen mustards – see Chapter 1) or by attack at guanine-O6 (Figures 2.11).

(In addition, there is a lesser reaction path that can contribute to the toxicity of chloroethylnitrosoureas: they can alkylate the nitrogen at position 3 of adenine, forming alkylated adenines, which can be removed by a specific DNA repair enzyme, called alkyladenine glycosylase (Li et al., 2003; Matijasevic et al., 1991).)

Before explaining how the crosslink forms, however, a few words about the right half of the cleaved molecule (blue arrows in Figure 2.11), which generates reactive isocyanates that can react with and damage many proteins (Cheng et al., 1972; Montgomery et al., 1967), including some involved in DNA repair (Ali-Osman et al., 1985; Kann, 1978; Kann et al., 1974; Wheeler et al., 1975). The commonly used BCNU/carmustine and CCNU/lomustine produce these extraneous and potentially harmful carbamoylation reactions. Although there are chloroethylnitrosoureas that do not produce isocyanates (Dive et al., 1988), they have not been developed, because of the clinical disillusionment with chloroethylnitrosoureas in general (Kohn, 1981).

We return now to the reaction pathway leading from attack at guanine-O6 to the production of guanine:cytosine crosslink (Tong et al., 1982) (Ludlum, 1997). This pathway is important, because it can be blocked in cells that have active MGMT; cancer cells deficient in this enzyme are highly vulnerable to being killed by the chloroethynitrosoureas (Sariban et al., 1987).

The reactions via the GO6 alkylation pathway leading to the production of DNA crosslinks between guanine and its paired cytosine are explained in Figures 2.11 and 2.12. In brief, the chloroethyl group ($ClCH_2CH_2$-) adds to (alkylates) a guanine at the guanine-O6 position. At this point, the MGMT repair enzyme can remove that chloroethyl group to regenerate a perfectly normal guanine. Competing with that repair reaction, the chloroethyl group can react with a nitrogen in the guanine ring to produce a new 5-membered ring. The new 5-membered ring then opens and leads to the G:C crosslink. Unless the crosslink is repaired by other DNA repair processes, the cell is likely to die.

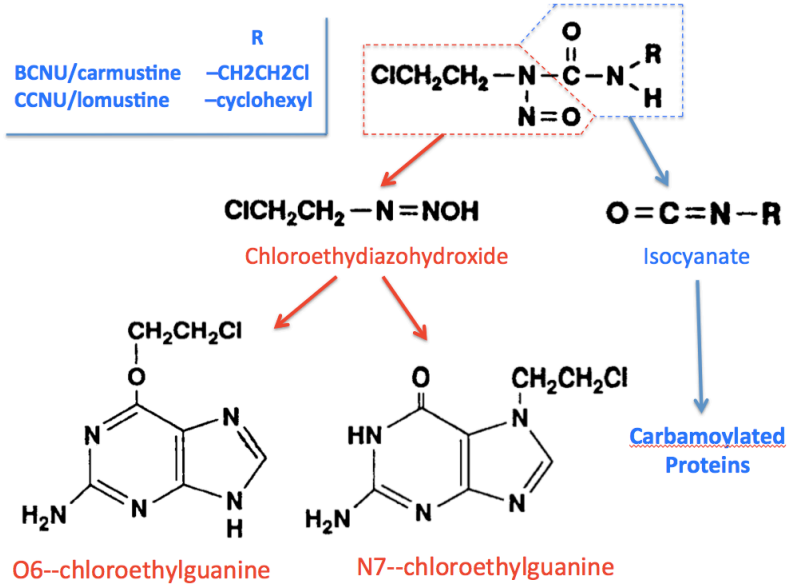


Figure 2.11. Reactions of the chloroethylnitrosoureas (BCNU/carmustine and CCNU/lomustine. The molecule spontaneously breaks into 2 pieces: chloroethyldiazohydroxide and an isocyanate. The former (red) chloroethylates DNA, mainly at guanine-O6 and guanine-N7. The latter (blue) binds to proteins and inactivate enzymes, and could be a source of toxicity.

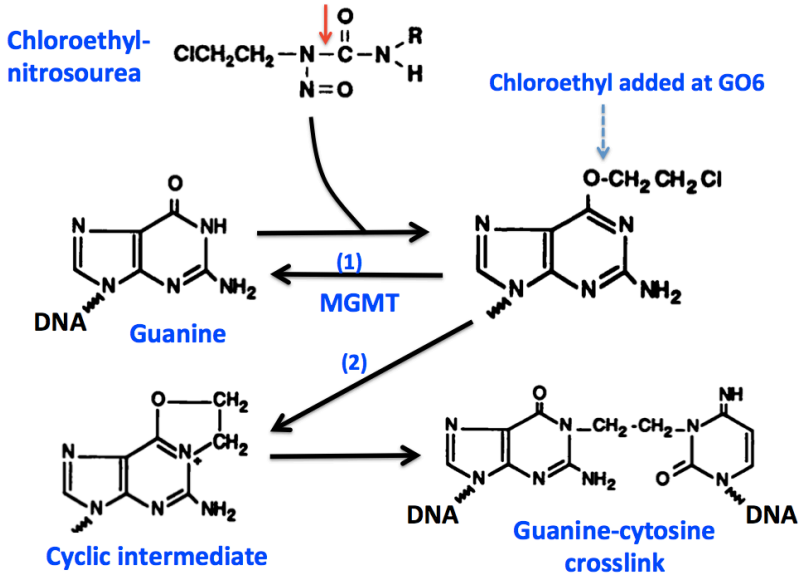


Figure 2.12. This scheme shows how chloroethylnitrosoureas crosslink between the guanine and cytosine in a DNA base pair and how the repair enzyme, MGMT, prevents that from happening. Chloroethylnitrosourea (top) spontaneously breaks to form a reactive intermediate (Figure 2.11) that adds a chloroethyl group to guanine-O6 (upper right), which can then undergo either of 2 reactions: (1) MGMT can remove the chloroethyl group to regenerate a normal guanine, which would

repair the DNA perfectly, or (2) the Cl can come off as the C to which it was attached binds to an N in the guanine ring, forming a new 5-membered ring (lower left), which can go on to react with the base-paired cytosine to form the crosslink on the lower right (Tong et al., 1982). The extent of crosslink formation depends on the balance between reactions (1) and (2). (See also Figure 2.11.)

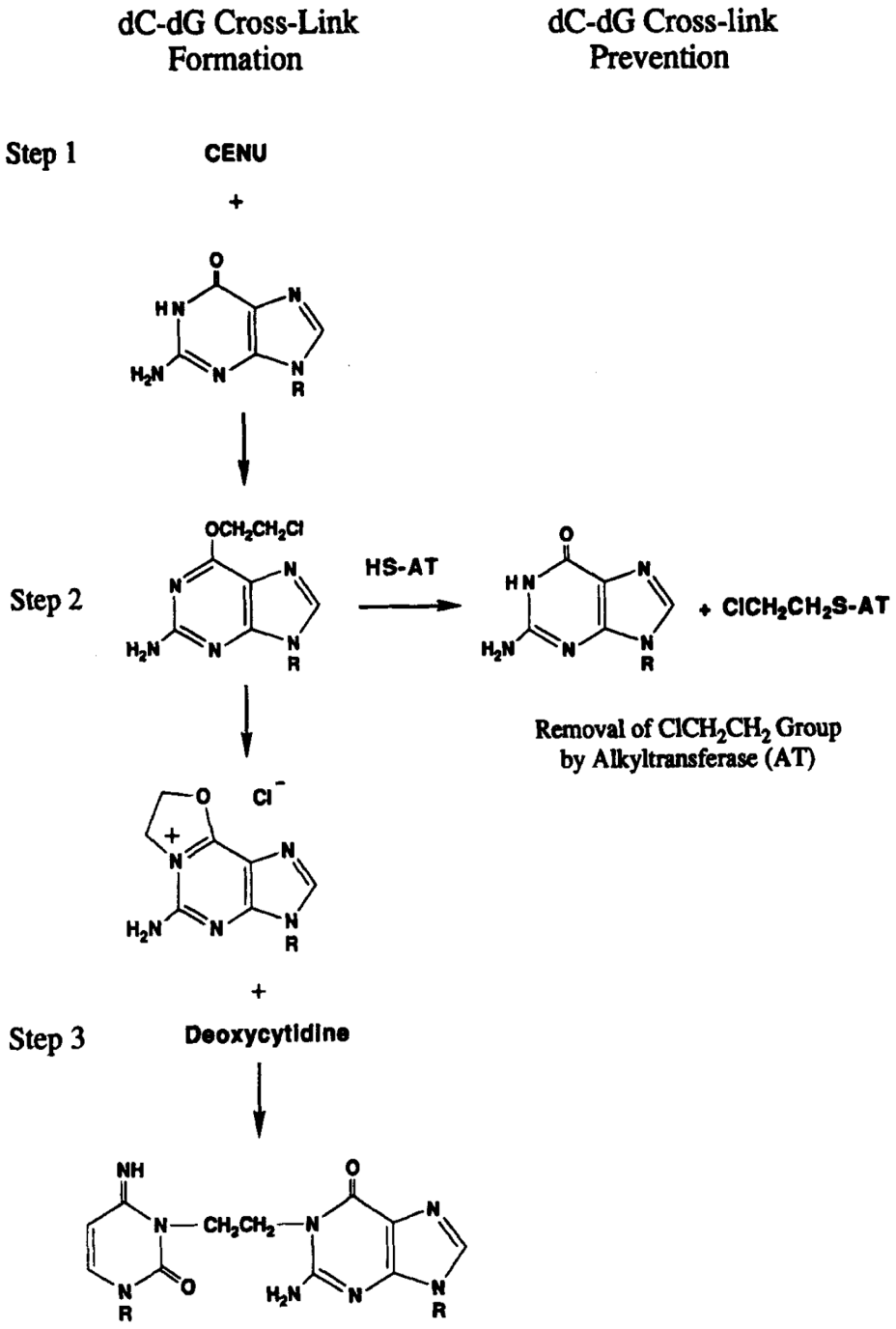


Figure 2.13. How chloroethylnitrosoureas form DNA interstrand crosslinks via the GO6 pathway, as determined and depicted by David Ludlum (Ludlum, 1997); this is another view of the chemistry in Figure 10, showing Ludlum's concept of the reaction steps. **Step 1:** the chloroethylnitrosourea (CENU) adds a CH_2CH_2Cl group to the oxygen at position 6 of a guanine in DNA. **Step 2:** the CH_2CH_2Cl group can be removed by alkyltransferase (AT, which another name for MGMT), thereby preventing crosslink formation; otherwise, the Cl comes off, and a new 5-membered ring forms on the guanine. **Step 3:** the transient 5-membered opens as a crosslink forms between the guanine and the cytosine of the base-pair. (R = deoxyribose of the DNA; "HS-AT" in Ludlum's diagram is to indicate the sulfhyryl (SH) group on AT that is the enzyme's reaction site.)

A nitrosourea targeted to a specific tissue: streptozotocin

Malignant tumors of the insulin-producing islets of the pancreas are rare. Something else rare about them, which makes them of special interest, is that there is a drug that targets this specific tissue. The drug is streptozotocin, a methylnitrosourea connected to a glucose moiety (Figure 2.14). Streptozotocin is made by a microorganism (a Streptomyces mold), perhaps evolved to kill competitor organisms that take up glucose as an energy-producing nutrient. The competing organism takes up streptozotocin, thinking it is taking up glucose, but, like a Trojan Horse, the streptozotocin proceeds to methylate the competitor's DNA and kills it. Aside from mitomycin (see Chapter 1), streptozotocin is the only other alkylating agent I know of that is made by an organism in nature. Notable also about the tissue selectivity of streptozotocin is that it is almost completely devoid of bone marrow toxicity (Moertel et al., 1977).

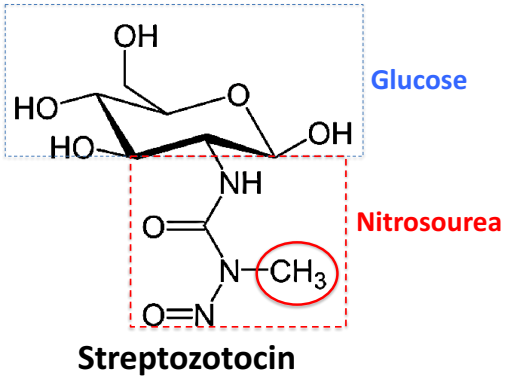


Figure 2.14. Streptozotocin consists of a glucose part that targets the drug to insulin-producing islet cells in the pancreas and to tumor cells arising from those islets. The nitrosourea part of the molecule then methylates guanine-O6 positions in DNA, which kills the cell. (The CH_3 in the nitrosourea part is the methyl group that is transferred to the guanine.)

Insulin-producing pancreatic islet cells take up glucose from the blood in order to regulate the rate of insulin production according to the blood glucose concentration. The glucose moiety of streptozotocin targets the drug to the islet cells and the methylnitrosourea part of the molecule kills them (Evans-Molina et al., 2007). In fact, streptozotocin causes diabetes by destroying normal islet cells. Some islet tumors (about 30% of patients) respond to treatment with streptozotocin (Moertel et al., 1994). The possible relationship between streptozotocin responsiveness and MGMT levels however seems not to have been investigated.

The concept of malignant pancreatic islet cell tumors that overproduce insulin ("insulinomas") was expanded to "pancreatic endocrine tumors," because some of those rare tumors produce other hormones than insulin. Streptozotocin in combination with other drugs is used to treat those tumors (Fjallskog et al., 2008; Moertel et al., 1992).

Chemists made a more potent streptozotocin variant, called chlorozotocin, in which the methyl group (circled red in Figure 2.14) was replaced by a chloroethyl, thereby conferring DNA crosslinking ability. On clinical trial, however, chlorozotocin, although more potent, was no better than streptozotocin at the optimum dose of each drug; hence, chlorozotocin was dropped from further study (Moertel et al., 1992).

Final word.

Evaluation of the MGMT DNA repair protein has become a useful predictor of response to DNA guanine-O6 targeted drugs, which makes it possible to avoid administering toxic chemotherapy in patients whose tumors will not respond to it.

The experience with chloroethylnitrosoureas shows the problems that arise in therapeutic agents that are highly reactive and that engage in many potentially toxic reactions. Their remarkable ability to cure tumors in mice however points to anti-cancer potential that may not have been fully tapped. Further effort may be worth while to find out why these drugs are so toxic in humans and to follow up on chemical modification that reduce the toxic side reactions.

Summary

Chapter 1 was about anticancer drugs that react (alkylate) at the N7 position of guanine in DNA. The current chapter was about more powerful alkylating drugs that attack also the guanine-O6 position. The most important of these was temozolomide, which became useful, especially in the treatment of brain cancer, because it was one

of the few drugs able to penetrates the blood-brain barrier and get into the brain. However, the guanine-O6 (GO6)-alkylating drugs were found to be effective only against cancers that lacked an enzyme (methylguanine-methyltransferase, MGMT) that would remove the GO6-alkylations before they could exert their cancer killing effects. Patients whose cancers had active MGMT received little or no benefit from those drugs, including temozolomide. Only patients in whose cancers MGMT genes were suppressed (by an epigenetic mechanism) benefited from these drugs. Another factor that came into play was the mismatch-repair system that detects and repairs base-pairs that do not match, *i.e.,* base-pairs other than G:C or A:T. The mismatch repair enzymes *increased* the cancer cell killing effect of temozolomide and related drugs against the MGMT-inactive cancers. Thus, the patients who received the most benefit from temozolomide were those whose cancers were both MGMT-inactive and mismatch repair active. In other words, if the mismatch repair system was inactive (due to mutation in one of its enzymes), then the drug was less effective, even against cancers whose MGMT was inactive. Therefore, MGMT and mismatch repair enzyme activities helped to predict how effective the GO6-alkylating drugs would be against a cancer in a particular patient.

Another class of GO6-targetted alkylating agents were the chloroethylnitrosoureas (carmustine (BCNU) and lomustine (CCNU)). These drugs were extraordinarily effective against mouse cancers, but disappointing against human cancers, in large part because their toxicities were difficult to manage. Like, temozolomide, they were most effective against cancers whose MGMT enzyme was inactive. Unlike temozolomide, however, they were able to produce inter-strand crosslinks in DNA. The chloroethylnitrosoureas were found to engage in very complicated chemical reactions that led to toxicity in addition to therapeutic potential. The possibility of modifying these drugs in a manner that would reduce their undesired reactions, however, was not fully explored.

It seemed that it might be possible to modify GO6-alkylating drugs in a manner that would allow them to enter certain cancer cells but not normal cells. A drug that suggested this possibility was streptozotocin, which consists of a glucose part linked to a methylnitorosurea part. The glucose part carried the drug into the islet cells of the pancreas and the methylnitorourea part then killed the cells. The drug was if fact useful in the treatment of the rare islet cell tumors of the pancreas. Thus, it seemed that chemical modifications of nitrosoureas might lead to new drugs for particular cancer types.

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